

Application No.: 10/734,936
Docket No.: CL1878 US NA

Page 6

Claim Amendments

Claim 1 (Currently Amended). A method for the directed integration of an expressible DNA fragment lacking a selectable marker into a bacterial chromosome of an *E. coli* comprising:

- a) providing at least one first recombination element having the general structure in the 5' to 3' direction:
5'-RR1-RS-SM-RS-RR2-3'; wherein
 - (i) RR1 is a first recombination region of about 10 to 50 bases;
 - (ii) RS is a recombination site responsive to a site-specific recombinase;
 - (iii) SM is a DNA fragment encoding a selectable marker; and
 - (iv) RR2 is a second recombination region of about 10 to 50 bases;
- b) providing at least one second recombination element having the general structure in a 5' to 3' direction:

X-RR3; wherein

- (i) X is an expressible DNA fragment having homology to the second recombination region; and
- (ii) RR3 is a third recombination element of about 10-50 bases;
- c) providing a recombination proficient bacterial host harboring a λ -Red recombinase system, having a bacterial chromosome comprising:
 - (i) a first chromosomal region having homology to said first recombination region;
 - (ii) a second chromosomal region having homology to said third recombination region;
- d) transforming said recombination proficient host with the first and second recombination elements, wherein both elements are integrated into the bacterial chromosome between the first and second chromosomal regions forming a construct having the general structure in the 5' to 3' direction;
5'-RR1-RS-SM-RS-RR2-X-RR3;
- e) selecting and isolating transformed hosts having the construct of (d) on the basis of the selectable marker;
- f) expressing a site-specific recombinase in the isolated hosts of (e) wherein the selectable marker is excised from the chromosome and whereby the expressible DNA fragment is inserted into the bacterial chromosome, lacking the selectable marker.

Claim 2 (Withdrawn). A method for the directed integration of an expressible DNA fragment lacking a selectable marker into a bacterial chromosome comprising:

- a) providing at least one first recombination element having the general structure in the 5' to 3' direction:

Application No.: 10/734,936
Docket No.: CL1878 US NA

Page 7

5'-RR1-RS-SM-RS-Y-RR2-3'; wherein

- (i) RR1 is a first recombination region of about 10 to 50 bases;
 - (ii) RS is a recombination site responsive to a site-specific recombinase;
 - (iii) SM is a DNA fragment encoding a selectable marker;
 - (iv) Y is a first expressible DNA fragment; and
 - (v) RR2 is a second recombination region of about 10 to 50 bases;
- b) providing at least one second recombination element having the general structure in a 5' to 3' direction:

5'-X-RR3-3'; wherein

- (i) X is a second expressible DNA fragment having homology to the second recombination region; and
 - (ii) RR3 is a third recombination region of about 10-50 bases;
- c) providing a recombination proficient bacterial host harboring a λ -Red recombinase system, and having a bacterial chromosome comprising:
- (i) a first chromosomal region having homology to said first recombination region;
 - (ii) a second chromosomal region having homology to said third recombination region;
- d) transforming said recombination proficient host with the first and second recombination elements, wherein both elements are integrated into the bacterial chromosome between the first and second chromosomal regions forming a construct having the general structure in the 5' to 3' direction;

5'-RR1-RS-SM-RS-Y-RR2-X-RR3;

- e) selecting and isolating transformed hosts having the construct of (d) on the basis of the selectable marker;
- f) expressing a site-specific recombinase in the isolated hosts of (e) wherein the selectable marker is excised from the chromosome and whereby the first and second expressible DNA fragments are inserted into the bacterial chromosome, lacking the selectable marker.

Claim 3 (Currently Amended). A method according to Claim 1 wherein either the first or second the-expressible DNA fragment is selected from the group consisting of regulatory elements, promoters, genes, coding sequences, and open reading frames.

Claim 4 (Previously Presented). A method according to Claim 1 wherein the site-specific recombinase is expressed by a gene residing on a plasmid.

Claim 5 (Previously Presented). A method according to Claim 1 wherein said first chromosomal region is upstream of a bacterial promoter.

Claim 6 (Previously Presented). A method according to Claim 1 wherein said first chromosomal region is upstream of an inter-operon chromosomal integration site.

Application No.: 10/734,936
Docket No.: CL1878 US NA

Page 8

Claim 7 (Original). A method according to Claim 3 wherein said expressible DNA fragment is a promoter selected from the group consisting of bacterial and phage promoters.

Claim 8 (Original). A method according to Claim 7 wherein said promoter comprises positive and negative regulatory sites for control of a regulatory circuit.

Claim 9 (Original). A method according to Claim 8 wherein said regulatory region comprises a *lac* operator site.

Claim 10 (Currently Amended). A method according to Claim 7 wherein said promoter is selected from the group consisting of ~~the a~~ phage T5 promoter, ~~the a~~ phage T7 promoter, and ~~the a~~ *lac* promoter.

Claim 11 (Currently Amended). A method according to Claim 1 ~~either one of Claims 1 or 2~~ wherein said selectable marker is selected from the group consisting of antibiotic resistance markers, enzymatic markers and amino acid biosynthesis enzymes.

Claim 12 (Canceled).

Claim 13 (Previously Presented). A method according to Claim 1 wherein said recombination sites are selected from the group consisting of *lox*, *frt*, *dif*, and *att*.

Claim 14 (Original). A method according to Claim 13 wherein said site-specific recombinase is selected from the group consisting of Cre, Flp, Xer, and Int.

Claim 15 (Currently Amended). A method according to Claim 1 ~~either one of Claims 1 or 2~~ wherein said recombination elements are generated by PCR.

Claim 16 (Previously Presented): A method according to Claim 1 wherein said recombination elements are from about 25 bases to about 4000 bases.

Claim 17 (Currently Amended). A method for the integration of a foreign promoter in place of a bacterial chromosomal promoter in a recombination proficient host cell of an *E. coli* comprising:

- a) providing at least one first recombination element having the general structure in the 5' to 3' direction:
5'-RR1-RS-SM-RS-RR2-3'; wherein
 - (i) RR1 is a first recombination region of about 10 to 50 bases;
 - (ii) RS is a recombination site responsive to a site-specific recombinase;
 - (iii) SM is a DNA fragment encoding a selectable marker; and
 - (iv) RR2 is a second recombination region of about 10 to 50 bases;
- b) providing at least one second recombination element having the general structure in a 5' to 3' direction:
5'-FP-RR3-3'; wherein
 - (i) FP is a promoter foreign to the recombination proficient host cell having homology to the second recombination region; and
 - (ii) RR3 is a third recombination element of about 10-50 bases;

Application No.: 10/734,936
Docket No.: CL1878 US NA

Page 9

- c) providing a recombination proficient bacterial host harboring a λ -Red recombinase system, having a bacterial chromosome comprising:
 - (i) a first chromosomal region upstream of a bacterial promoter having homology to said first recombination region;
 - (ii) a second chromosomal region, downstream of said bacterial promoter having homology to said third recombination region;
- d) transforming said recombination proficient host with the first and second recombination elements, wherein both elements are integrated into the bacterial chromosome between the first and second chromosomal regions forming a construct having the general structure in the 5' to 3' direction:
5'-RR1-RS-SM-RS-RR2-FP-RR3;
- e) selecting and isolating transformed hosts having the construct of (d) on the basis of the selectable marker;
- f) expressing a site-specific recombinase in the isolated hosts of (e) wherein the selectable marker is excised from the chromosome and whereby the foreign promoter is inserted into the bacterial chromosome in place of the bacterial promoter.

Claim 18 (Withdrawn). A method for the integration of an unlinked foreign promoter and foreign open reading frame into a bacterial chromosome in a recombination proficient host cell comprising:

- a) providing at least one first recombination element having the general structure in the 5' to 3' direction:
5'-RR1-RS-SM-RS-FP-RR2-3'; wherein
 - (i) RR1 is a first recombination region of about 10 to 50 bases;
 - (ii) RS is a recombination site responsive to a site-specific recombinase;
 - (iii) SM is a DNA fragment encoding a selectable marker;
 - (iv) FP is a promoter foreign to the recombination proficient host cell; and
 - (iv) RR2 is a second recombination region of about 10 to 50 bases;
- b) providing at least one second recombination element having the general structure in a 5' to 3' direction:
5'-FO-RR3-3'; wherein
 - (i) FO is an open reading frame foreign to the recombination proficient host cell having homology to the second recombination region; and
 - (ii) RR3 is a third recombination of about 10-50 bases;
- c) providing a recombination proficient bacterial host harboring a λ -Red recombinase system, having a bacterial chromosome comprising:

Application No.: 10/734,936

Docket No.: CL1878 US NA

Page 10

- (i) a first chromosomal region upstream of a bacterial intra-operon chromosomal integration site having homology to said first recombination region;
- (ii) a second chromosomal region, downstream of said bacterial intra-operon chromosomal integration site having homology to said third recombination region;
- d) transforming said recombination proficient host with the first and second recombination elements, wherein both elements are integrated into the bacterial chromosome between the first and second chromosomal regions forming a construct having the general structure in the 5' to 3' direction;
5'-RR1-RS-SM-RS-FP-RR2-FO-RR3;
- e) selecting and isolating transformed hosts having the construct of (d) on the basis of the selectable marker;
- f) expressing a site-specific recombinase in the isolated hosts of (e) wherein the selectable marker is excised from the chromosome and whereby the foreign promoter and foreign open reading frame are inserted into the bacterial chromosome.

Claim 19 (Withdrawn). A method for the integration of a foreign gene comprising a regulatory region and foreign open reading frame into a bacterial chromosome in a recombination proficient host cell comprising:

- a) providing at least one first recombination element having the general structure in the 5' to 3' direction:
5'-RR1-RS-SM-RS-FG-RR2-3'; wherein
 - (i) RR1 is a first recombination region of about 10 to 50 bases;
 - (ii) RS is a recombination site responsive to a site-specific recombinase;
 - (iii) SM is a DNA fragment encoding a selectable marker;
 - (iv) FG is a gene comprising a regulatory region, foreign to the recombination proficient host cell; and
 - (iv) RR2 is a second recombination region of about 10 to 50 bases;
- b) providing at least one second recombination element having the general structure in a 5' to 3' direction:
5'-FO-RR3-3'; wherein
 - (i) FO is an open reading frame foreign to the recombination proficient host cell having homology to the second recombination region; and
 - (ii) RR3 is a third recombination of about 10-50 bases;
- c) providing a recombination proficient bacterial host harboring a λ -Red recombinase system, having a bacterial chromosome comprising:

Application No.: 10/734,936
Docket No.: CL1878 US NA

Page 11

- (i) a first chromosomal region upstream of a bacterial intra-operon chromosomal integration site having homology to said first recombination region;
- (ii) a second chromosomal region, downstream of said bacterial intra-operon chromosomal integration site having homology to said third recombination region;
- d) transforming said recombination proficient host with the first and second recombination elements, wherein both elements are integrated into the bacterial chromosome between the first and second chromosomal regions forming a construct having the general structure in the 5' to 3' direction;
5'-RR1-RS-SM-RS-FG-RR2-FO-RR3;
- e) selecting and isolating transformed hosts having the construct of (d) on the basis of the selectable marker;
- f) expressing a site-specific recombinase in the isolated hosts of (e) wherein the selectable marker is excised from the chromosome and whereby the foreign promoter and foreign open reading frame are inserted into the bacterial chromosome.

Claim 20 (Previously Presented). A method according to Claim 17 wherein the site-specific recombinase is expressed by a gene residing on a plasmid.

Claim 21 (Original). A method according to Claim 17 wherein said promoter is selected from the group consisting of bacterial and phage promoters.

Claim 22 (Original). A method according to Claim 21 wherein said promoter comprises positive and negative regulatory sites for control of regulatory circuit.

Claim 23 (Original). A method according to Claim 22 wherein said regulatory region comprises a *lac* operator site.

Claim 24 (Currently Amended). A method according to Claim 21 wherein said promoter is selected from the group consisting of the a phage T5 promoter, the a phage T7 promoter, and the a *lac* promoter.

Claim 25 (Canceled).

Claim 26 (Previously Presented). A method according to Claim 17 wherein said recombination sites are selected from the group consisting of *lox*, *frt*, *dif*, and *att*.

Claim 27 (Original). A method according to Claim 26 wherein said site-specific recombinase is selected from the group consisting of Cre, Flp, Xer, and Int.

Claim 28 (Original). A method according to any one of Claims 17-19 wherein said recombination elements are generated by PCR.

Claim 29 (Previously Presented). A method according to Claim 17 wherein said recombination elements are from about 25 bases to about 4000 bases.

Application No.: 10/734,936
Docket No.: CL1878 US NA

Page 12

Claim 30 (Previously Presented). A method according to any of Claims 1, or 17 wherein steps (d) – (f) are repeated one or more times.